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Microsatellites for *Tetracentron sinense* (Trochodendraceae), a Tertiary relict endemic to East Asia[1]

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- *Premise of the study: Tetracentron sinense* (Trochodendraceae) is a Tertiary relict endemic to East Asia. Microsatellite markers were developed and characterized to investigate the population genetics of the species.
- *Methods and Results:* Microsatellite markers were isolated from the genome of *T. sinense* using the protocol of Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO). Eight polymorphic microsatellite markers were assessed in 44 samples collected from three wild populations. The number of alleles observed for each locus ranged from two to five. The observed and expected heterozygosities ranged from 0.0000 to 0.9375 and 0.0000 to 0.7681, respectively.
- *Conclusions:* The microsatellite markers will be helpful in further studies of the population genetics and phylogeography of *T. sinense*.

Key words: microsatellite; polymorphism; population genetics; *Tetracentron sinense*; Trochodendraceae.

Tetracentron Oliv. (Trochodendraceae) is one of the earliest branching eudicots (Angiosperm Phylogeny Group, 2009). Considerable fossil evidence suggests that the genus was widely distributed in North America, Europe, and northeastern Asia during the Tertiary (Manchester et al., 2009). The genus is currently endemic to East Asia, including central to southwestern China, northern Vietnam, northern Burma, northeastern India, Bhutan, and eastern Nepal. *Tetracentron sinense* Oliv. is the only extant species of the genus, and grows along streams and forest margins in evergreen broad-leaved forests and mixed evergreen-deciduous forests, at elevations of 1100–3500 m (Fu and Bartholomew, 2001). Unfortunately, due to excessive logging and its poor natural regeneration, it currently only exists in remote mountains, canyons, cliffs, or steep slopes and has island-like distribution; it has been treated as an endangered species in China (Fu, 1992).

As a Tertiary relict, *T. sinense* provides us an ideal system to perform phylogeographical investigations to sketch the refugia of the Late Tertiary/Quaternary glaciations in East Asia, especially in the eastern Himalayas, an important center of diversity and distribution for Tertiary relicts and endemic plants. This knowledge will help to deepen our insights into the evolutionary history of these elements endemic to East Asia. The objective of this research was to develop a set of polymorphic microsatellite markers for *T. sinense*.

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METHODS AND RESULTS

Microsatellite markers for *T. sinense* were developed following the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) protocol (Zane et al., 2002). Total genomic DNA was extracted from the silica gel–dried leaves using a cetyltrimethylammonium bromide (CTAB) methodology. The DNA derived was digested by an *Mse*I restriction enzyme (New England Biolabs, Ipswich, Massachusetts, USA) and then ligated to the *Mse*I adapter pair (5′-TACTCAGGACTCAT-3′/ 5′-GACGATGAGTCCTGAG-3′) with T4 DNA ligase (Fermentas, Hamilton, Ontario, Canada) at 37°**C** for 2 h. The diluted products were amplified with the primers *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) according to the following PCR program: 95°C for 3 min; followed by 20 cycles of 94°C for 30 s, 53°C for 60 s, and 72°C for 60 s; and a final extension step at 72°C for 8 min. The PCR products were purified using a gel extraction kit (Sangon, Shanghai, China), then the fragments within the size range of 250–800 bp were hybridized with two kinds of 5′-biotinylated probes $(AC)_{15}$/$(AG)_{15}$ at 48°**C** for 2 h, respectively, to enrich the fragment containing microsatellite repeats. We used streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA) to separate and capture the DNA fragments hybridized to the probes at room temperature for 50 min, and then two washing steps followed, including three washes in TEN_{1000} for 24 min, and three washes in the mixed solution (500 μL 0.2× saline sodium citrate [SSC] and 0.1% sodium dodecyl sulfate [SDS]) for 15 min. Microsatellite-enriched DNA fragments were amplified with the *Mse*I-N primer with the PCR program mentioned above (30 cycles). PCR fragments were purified, then ligated into pGEM-T vector (Promega), and transformed into Trans1-T1 Phage Resistant Chemically Competent Cells (Quanshijin, Beijing, China). The positive clones were picked out by blue-white screening and tested by PCR using the primer pairs $(AG)_{10}$/$(AC)_{10}$ and $M13^+$ (5′-GTAAACGACGGCCAG-3′), respectively. In total, 196 clones with positive inserts were sequenced, and 108 of them were found to contain microsatellite repeats by SSRIT software (http://www.gramene.org/db/searches/ssrtool/). Primers were designed for 64 sequences using Primer Premier version 5.0 (http://www.premierbiosoft.com/) and were synthetized by BGI (Shenzhen, Guangdong, China). Initially, polymorphisms of these putative loci were scanned in 12 individuals of *T. sinense* from six natural populations. Loci displaying polymorphism were amplified to detect the genotypes of 44 individuals of *T. sinense* from three wild populations, including: (1) Zhushan population (16 individuals), Zhushan County, Hubei, China; (2) Jinfoshan population (12 individuals), Nanchuan District, Chongqing, China; and (3) Leibo population

TABLE 1. Characterization of 19 microsatellite primers for *Tetracentron sinense*.

Locus	Primer sequences (5′–3′)	Repeat motif	Expected length (bp)	T_a (°C)	GenBank accession no.
Ts09	F: GGAGCGTAATTCTAGCACTT R: GCCTACTGAACCCGAATCTA	$(TG)_4$	184	58	JN865215
Ts13	F: TCTTCCCTCCTTTCTTGTTC R: CCTTCCCATTTGGTTCATC	$(GT)_5$	119	54	JN865216
Ts16	F: GATCGAGGACCACCATAA R: AACAACAGAAACCCAGGAG	$(GA)_9$	101	59	JN865217
Ts17	F: ACACGGACTCCATTTGACA R: AATGTGCGGAAGCGTAA	$(AG)_8$	256	58	JN865218
Ts19	F: CACGGGATGGGTAGAACTT R: CATAGAATACGGACCGAACA	$(GA)_7$	212	59	JN865219
Ts22	F: TCCATCACGCCTCAAACA R: CGAAGTTACGGTCAGGAAA	$(TG)_8A(GT)_6$	120	58	JN865220
Ts28	F: TGCTGGGCTTGTCTTCT R: GTGTCATACATCAGCGTGT	$(CA)_{15}$	139	57	JN865221
Ts30	F: ATACATCGAGACCTCTAAGC R: GGAGTGCAGTGAACATAAAC	$(TC)_3T(TCC)_2(TC)_4$	145	58	JN865222
Ts31	F: AATAACCACTGGCTTGAATG R: CTGGAGCAGAAATCTGTCTTA	$(CTCTTT)_3$	211	58	JN865223
Ts33	F: GAATCCGTTTGTTGTGAAGG R: CTTGCCACATCCCACTC	$(AGA)_2G_3(GA)_3$	187	56	JN865224
Ts34	F: GCTCAATGGGTGGACAA R: GCCTGAGCCCATTTGTAT	$(CT)_{10}T(TC)_7$	166	56	JN865225
Ts40	F: GTGGTCTAAACATCGCCTCT R: GACAACCATAATGCCTTTCC	$(AG)_5$	192	59	JN865226
Ts43	F: TCTCCATCCGGGAGTATTT R: AGGGCAGTTGTTGTCCTTT	$(AG)_8$	177	56	JN865227
Ts45	F: GTAACCATATTGGGTCTTTG R: CGAGCATACTTGGCACT	$(GA)_9AAA(AG)_8$	116	58	JN86528
Ts46	F: TCACCCTGTAAACCCATAA R: GCTTGCACAAAGCCATTA	$(AG)_{11}$	179	59	JN865229
Ts48	F: ATCATCTCCAGAACTCCCTC R: GGCAACATTAACCACCACAT	$(TC)_{10}$	268	59	JN865230
Ts49	F: GGTCAACTGGGAAATAGC R: ATTGGTGAATTTGGTGC	$(AG)_6T(GA)_6$	135	58	JN865231
Ts54	F: GGTTGGGTTGCCTACGT R: TCAATCCCTCTGCACATT	$(GT)_8$	178	59	JN865232
Ts59	F: ATGTGGTTACGGCCTACT R: CCAACCCAAACTCATTCA	$(TG)_5$	218	59	JN865233

Note: T_a = annealing temperature.

(16 individuals), Leibo County, Sichuan, China (Appendix 1). The PCR reactions were performed in a 25-μL reaction mixture with 2.5 μL of 10× PCR buffer (100 mmol L^{-1} Tris-HCl [pH 8.3], 500 mmol L^{-1} KCl, and 15 mmol L^{-1} $MgCl_2$), 0.5 μL of dNTP (2.5 μmol each), 0.5 μL of each primer (5 μmol L^{-1}), 0.3 μL of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, Liaoning, China), and 1.2 μL of genomic DNA. PCR amplifications were conducted in a PTC-200 thermal cycler (BIO-RAD, Foster City, California, USA) under the following conditions: 93°C for 3 min; followed by 30–38 cycles at 93°C for 30 s, at the optimized annealing temperature (Table 1) for 30 s, and at 72°C for 1 min; and a final extension step at 72°C for 7 min. The PCR products were separated in 8% sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) using a 25-bp ladder (25–500 bp) molecular size standard and then visualized by silver staining. Features of each microsatellite locus, including the number of alleles (*A*), expected heterozygosity (H_e), observed heterozygosity (H_o), and deviations from Hardy–Weinberg equilibrium (HWE) were calculated in GENEPOP version 3.4 software (http://genepop.curtin.edu.au/). The linkage disequilibrium tests between loci were calculated in FSTAT version 2.9.3, with significance levels being calibrated by Bonferroni correction.

The results indicated that 19 of 64 screened primers generated specific amplification with the expected lengths (Table 1). Among them, eight loci displayed polymorphism with the number of alleles per locus ranging from two to five (Table 2). H_o and H_e ranged from 0.0000 to 0.9375 and 0.0000 to 0.7681, respectively. In this study, there are neither loci deviating remarkably

TABLE 2. Results of initial primer screening in three populations of *Tetracentron sinense.*

	Zhushan, Hubei, China			Jinfoshan, Chongqing, China			Leibo, Sichuan, China		
Locus	*A*	H_o	H_e	*A*	H_o	H_e	*A*	H_o	H_e
Ts9	1	0.0000	0.0000	2	0.4167	0.4891	2	0.4375	0.5141
Ts13	1	0.0000	0.0000	2	0.0000	0.4638	2	0.6250	0.5161
Ts19	2	0.3125	0.4657	2	0.0833	0.5181	2	0.2500	0.5081
Ts30	2	0.9375	0.5141	2	0.4167	0.3442	2	0.8750	0.5081
Ts40	2	0.1875	0.4980	1	0.0000	0.0000	2	0.3750	0.3871
Ts43	3	0.3125	0.6472	2	0.0833	0.4891	2	0.0625	0.4173
Ts45	3	0.1250	0.5565	5	0.5000	0.7681	4	0.7500	0.6996
Ts59	1	0.0000	0.0000	2	0.0833	0.5181	2	0.6250	0.4839

Note: *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity.

from HWE ($P < 0.01$) nor loci displaying significant linkage disequilibrium ($P < 0.05$) (Table 2).

CONCLUSIONS

We reported the eight polymorphic microsatellite loci developed in *T. sinense*. They will facilitate studies on the phylogeography of the species, which will improve knowledge on glacial refugium and the postglacial recolonization of relict plants in East Asia, especially in the eastern Himalayas. Furthermore, they will be useful tools in further studies on gene flow, mating system, and spatial patterns of genetic diversity of the plant.

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APPENDIX 1. Information on voucher specimens for *Tetracentron sinense*.

Population	Voucher no.	Herbarium	Collection date	Geographic coordinates	Elevation (m)	Habitat
Zhushan	JIYH09034	KUN	7 May 2009	32°31′29.55″N, 110°23′43.30″E	793	Deciduous broad-leaf forest
Jinfoshan	JIYH09041	KUN	20 May 2009	28°56′42.70″N, 107°09′9.66″E	815	Deciduous broad-leaf forest
Leibo	JIYH09084	KUN	5 July 2009	28°30′42.20″N, 103°36′37.20″E	1310	Deciduous broad-leaf forest